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Development of adenoviral vectors armed with TNF-related therapeutic proteins for gene therapy

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Chapter 7

Conclusions and future perspectives

In this thesis, we aimed to develop adenoviral vectors armed with TRAIL and RANKL proteins for their use as treatments for cancer or fibrosis and to use the CRISPR/Cas9 technology as a treatment for renal cell carcinoma by knocking out the EGFR.

Cancer is a complex disease, and its usual treatments tend to be toxic to healthy tissues. Therefore, several ligands from the Tumor Necrosis Factor (TNF) family came to the spotlight in the search for new anti-cancer therapeutics due to their tumoricidal activity by apoptosis induction. In **chapter two**, we describe an outlook of TNF- α , FasL, and TRAIL as cancer treatments in diverse preclinical and clinical stages and summarize how gene therapy may overcome the ligands' natural limitation, improving at the same time their effectiveness.

The use of adenoviral vectors as a method to deliver therapeutic genes is increasing. Recently, the use of the adenoviral vectors gained new attention due to their use in developing SARS-CoV-2 vaccines. However, despite the approval of SARS-CoV-2^{27,28} adenoviral-based vaccines, there are still drawbacks, such as off-target effects and unwanted host immune responses that need to be overcome.

In the production of virus-based gene therapy medicines, purification is a pivotal step. Therefore, in **chapter three**, we sought to develop a rapid and straightforward purification process by employing anion exchange chromatography together with an ultrafiltration step. The small-scale purification gave titers in the order of 10^{10} plaque-forming units per ml (pfu/mL) with high purity, similar to the standard CsCl gradient purification. This methodology is suitable for scale-up; the vectors can be used for *in vivo* studies after assessing host cell contaminants' presence (e.g., proteins and genetic material) and endotoxins¹.

EGFR has been studied as a target for cancer therapy in the last decades due to its involvement in cell proliferation and cell survival in diverse malignancies. The most common approaches to target EGFR are monoclonal antibodies (mAbs) and small-molecules tyrosine kinase inhibitors (TKIs)². The mAbs target the receptor's extracellular domain, while the TKIs target only the TK domain blocking the downstream signaling³. In **chapter four**, we delete the EGFR in renal cell carcinoma using the CRISPR/Cas9 technology, which allows us to resemble the treatment with TKI. However, by using CRISPR/Cas 9, all functions of

EGFR can be removed; this may lead to the activation of alternative pathways⁴. Additional studies are required to elucidate the discrepancy and correlation between the use of EGFR inhibitors and knockout.

TRAIL is a promising protein for cancer treatment because it can selectively induce apoptosis in cancer cells by binding to death receptors⁵. Therefore, we developed three adenoviral vectors to improve TRAIL apoptotic activity and overcome its short half-life (60 min) in blood circulation. In **chapter five**, we assessed the apoptotic activity via bystander effect of the adenoviral-expressed scFv425-sTRAIL, scFv425-DHER (DR5-specific), and scFv425-4C7 (DR4-specific) fusion proteins. The fusion proteins affected the cell viability by inducing apoptosis in different cancer cell lines. Although we did not see any significant difference between the activities of scFv425-sTRAIL, scFv425-DHER, and scFv425-4C7, we did see a higher apoptotic effect induced by the fusion proteins than in the one induced by the Rh-sTRAIL variants alone or in combination with mAb 425 (anti-EGFR). These results are consistent with previous studies showing that sTRAIL regains its membrane-bound-like activity when present in a fusion protein^{6,7}. Therefore, it is worth exploring these adenoviral systems combined with compounds that can increase DR4 or DR5 expression while simultaneously sensitizing the cells towards TRAIL apoptosis activity by downregulating anti-apoptotic proteins^{8,9}.

Fibrosis is a currently untreatable disease. An excess of extracellular matrix deposition (ECM) in fibrosis results from the abnormal wound-healing response presence in diverse injury tissues. An approach to aid fibrosis regression is the degradation of ECM; this can be done via matrix metalloproteinases (MMPs) such as MMP-9^{10,11}; the production of MMP-9 can be induced by RANKL/RANK pathway. In **chapter six**, we hypothesized that RANKL could stimulate RANK on macrophages and trigger ECM degradation in fibrotic conditions. However, to minimize the effect of RANKL in normal tissues and overcome its short half-life, we have constructed replication-defective adenoviral vectors (Ad-RANKL WT and Ad-RANKL Q236D). These vectors showed long-term functional expression of adenoviral-expressed RANKL_WT and RANKL_Q236D proteins. Adenoviral-produced RANKL_Q236D activated the NF- κ B pathway in RAW 264.7 cells and avoided inhibition by exogenous OPG. The generation of Ad-RANKL Q236D may be a powerful new tool for studying and treating inflammation and fibrosis; it is worth evaluating it in *in vivo* studies.

In conclusion, our work shows that gene therapy using TNF-related proteins is a promising therapeutic tool that may help treat and understand diverse diseases such as fibrosis and cancer.

TNF- α , FasL, and TRAIL can induce apoptosis by activating the extrinsic pathway when they bind to the death receptors, making them useful as anti-cancer therapeutics¹². At the same time, RANKL was initially known for its involvement in bone homeostasis and immune regulation¹³ and subsequently known for its participation in cell proliferation in cancer¹⁴. However, the TNF ligands may be effective against other diseases; for instance, TRAIL's administration may protect against nonalcoholic fatty liver disease in type 2 diabetes¹⁵ and atherosclerosis¹⁶ in mice. Moreover, RANKL administration can benefit bone marrow transplants by helping to regenerate the thymus¹⁷ and orthodontic treatments by accelerating the tooth movement¹⁸. Likewise, the use of TNF ligands together with adenoviral vectors may circumvent issues such as delivery, specificity, and short half-life^{16,19}.

Although the Ad.scFv425-sTRAIL/DHER/4C7 were tailor specifically for cancer treatment and Ad. RANKL WT/Q236D were designed to treat fibrosis. Nevertheless, these types of vectors could be used to study and treat other diseases alone or in combination with other drugs; for example, viral vectors armed with TRAIL might help to reduce the development of cardiomyopathies²⁰ and helped mitigate atherosclerosis¹⁶ in mouse diabetic models. Similarly, studies in mice showed that administration of RANKL using a viral vector might be beneficial for RANKL-dependent autosomal recessive osteopetrosis (ARO), a genetic disease mainly characterized by an increase in bone density^{21,22}. Furthermore, the specificity of these vectors can be modified and/or improved by using cancer-specific (e.g., human telomerase [hTERT] and human epidermal growth factor receptor/neu [HER2/NEU]) or tumor-specific (Mucin 1 [MUC1], breast cancer 1 [BRCA1]) promoters²³; and by modifying the knob and fiber domains of the adenoviral vectors²⁴.

We treated a renal cell carcinoma cell line with CRISPR/Cas 9 to knock out the EGFR. The resulting RC21 EGFR^{-/-} cell line helped us assess the specificity of the apoptotic activity of our three adenoviral-expressed TRAIL-anti EGFR fusion proteins. Further studies may help elucidate alterations in the proteome and transcriptome of RC21 consequent to the EGFR ablation and to understand how those alterations may or may not benefit cancer therapy.

In conclusion, CRISPR/Cas9 enabled the development of a model that may help to understand diseases²⁵. In addition, using CRISPR/Cas9 for genome editing can be used for screening, identifying "key" genes involved in disease progression, and studying drug-gene interactions²⁶. Thus, all these characteristics of CRISPR/Cas9 show its versatility as a gene therapy tool.

Overall, our work shows that gene delivery via adenoviral vectors may result in the constant production of recombinant proteins, such as TRAIL and RANKL. It may allow prolonged production, thus avoiding systemic toxicity or undesired effects in healthy tissues leading to potential novel treatments for cancer, fibrosis, inflammatory diseases, osteoporosis, and other diseases. However, further efficacy and safety studies are still required.

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